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Application of the Multiscreen System to Cytokine Radioreceptor Assays

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ABSTRACT

Radioreceptor assays are becoming increasingly valuable in the biotechnology community for a variety of basic and applied research applications. It is clear, for example, that assessing the potential spectrum of biological activities of a novel polypeptide regulatory factor can be greatly simplified by the development of a rapid radioreceptor assay, since a wide variety of cell types can be screened using a single type of assay. By contrast, searching for potentially diverse biological effects can be an extremely time-consuming process. In addition, screening for agonists/antagonists for hormones using radioreceptor assays has a marked advantage compared with biological assays, in that compounds or natural products that are toxic to cells will not read out as false positives in a binding assay. Our laboratory has developed a major program centered on the molecular characterization of receptors for polypeptide hormones involved in immune regulation, including a number of cytokines/interleukins and also several colony stimulating factors. We have developed a variety of radioreceptor- and fluorescence-based assay systems for ligand-receptor interactions, with applications in basic characterization, purification, cDNA cloning, and drug development screens for cytokine receptors. In this report we compare two assay formats, a standard phthalate oil centrifugation method and a novel plate filtration system, using the interaction between interleukin-1α and its receptor as a test system.

INTRODUCTION

In a general sense, the dogma that polypeptide hormones act on cells via specific cell surface receptors is well established. It is presumed that such small globular protein hormones cannot themselves cross the plasma membrane, unlike steroid hormones, and cannot effectively gain entry to cells via pinocytic and endocytic pathways, as these generally involve lysosomal delivery and, likely, protein degradation. Rather, polypeptide hormones bind to integral membrane proteins or complexes of proteins which contain, in addition to an extracellular ligand-binding region, an intracellular region capable of being activated by hormone binding, and hence delivering a signal.

Receptor charcterization is thus crucial to understanding the mechanism of action of a polypeptide hormone on target cells and tissues, and from the perspective of applied research and development, such an understanding is crucial to any rational attempts to control and modulate such processes using drugs. One group of polypeptide hormones which play a central role in the regulation of immune and inflammatory responses and which are, thus, of major biomedical importance are the cytokines or interleukins. Over the past five years, rapid advances have been made in our understanding of the structure and function of cytokine receptors (4). Thus, cDNA clones have been isolated for the IL-1 receptor (23), both subunits of the IL-2 receptor (3,11,12,20), the IL-4 receptor (19), and the IL-6 receptor (25). For the IL-1 receptor and the p55 subunit of the IL-2 receptor, cell-free radioreceptor binding assays have been developed, using soluble ligand binding fragments of the receptors (9,13). It is rapidly becomming clear that an understanding of the structure-function relationships of cytokine receptors is central to any attempts to manipulate these systems pharmacologically, and that it is

important for this purpose to constantly refine the assay methods available to us for studying ligand binding. In this report we describe the use of a novel plate filtration binding assay method to analyze the binding of IL-1 to its receptor, a ligand receptor system with which we have had considerable experience in our laboratory (5-8,16,22).

METHODS

Preparation of Cells, Cell Extract and Soluble IL-1 Receptor

EL-4 6.1c10 cells, a subclone of the EL-4 murine thymoma cell line, were propagated in suspension culture as described previously (17). For radioreceptor binding assays, cells were harvested by centrifugation, washed once with binding medium (see below), an aliquot counted in the presence of trypan blue to determine cell concentration and viability, and resuspended at the working stock concentration. Cell extracts were prepared as described (24). Briefly, cells were sedimented for 10 min at 2,000 x *g* to form a packed pellet (*ca.* 8 x 10^8 cells/ml), to which was added an equal volume of 1% Triton X-100 and a mixture of protease inhibitors. The cells were mixed with detergent by vigorous vortexing, and the extraction mix was incubated on ice for 15 min. At the end of this time the mixture was centrifuged at 11,000 x *g* for 30 min to remove debris, and stored frozen at -70°C until further use. This procedure has been shown to yield 100% recovery of IL-1 binding activity from the cells, as determined by a solid-phase nitrocellulose dot blot binding assay (24). Soluble murine IL-1 receptor was produced from cDNA encoding residues 1-316 of the intact molecule (9,23) by expression in HeLa cells, as described previously (9). The receptor protein produced in the system is designed to terminate just prior to the single membrane-spanning region, and is, hence, secreted into the medium. The protein was purified by affinity chromatography on IL-1RM5 Affigel (Bio-Rad, Richmond, VA), essentially as described by Dower et al. (9), and stored as a 50-µg/ml stock solution in phosphate (0.05 M, pH 7.4) buffered saline (0.15 M) (PBS).

Preparation of IL-1α, Radiolabeled IL-1α and IL-1RM5 Anti-IL-1 Receptor Antibody Affinity Matrix

Polypeptides corresponding to the naturally processed and released C-terminal fragments of the IL-1α and IL-1β precursors were expressed in *E. coli* and purified as described elsewhere (15). The purified protein was stored at -70°C as a 3-mg/ml solution in phosphate buffered saline. For preparation of radioligand, 30 µg aliquots from the stock solution were labeled with ^{125}I-sodium iodide, using the chloramine-T method (7). The labeled protein was immediately diluted into binding medium (RPMI 1640, 1% w/v bovine serum albumin, 0.1% w/v sodium azide, 20 mM HEPES pH 7.4) as a 30-nM stock solution, to minimize radiolysis. Routinely, the radioligand had a specific activity of 1-3 x 10^{15} cpm/mmol (*ca.* 1 atom iodine per molecule protein). The rat IgG2a monoclonal antibody against the murine IL-1 receptor (IL-1RM5) was derived, produced and purified as described elsewhere (10). Cross-competition experiments demonstrate that the antibody does not inhibit binding of IL-1 to the receptor, and is therefore directed against an epitope not involved in IL-1 binding. Purified antibody was coupled to Affigel (AFFI-10) according to the manufacturer's instructions, and a final loading of 2.0 mg antibody/ml gel was obtained.

Radioreceptor Assays

Binding of IL-1α to intact cells was measured by two methods. The first was a phthalate oil centrifugation assay. Briefly, cells at various concentrations were incubated with varying concentrations of ^{125}I-IL-1α in a total volume of 0.15 ml of binding medium for 2 h, in a refrigerator (8°C) on a rocker platform. Nonspecific binding was measured at the two highest concentration of radioligand using 10 µg/ml of unlabeled IL-1α to block receptors. At the end of the incubation, duplicate 60 µl aliquots of cell suspension were layered over 200 µl of a phthalate oil mixture (60% dibutyl-phthalate v/v, 40% bis-[2-ethyl-hexyl]-phthalate, v/v) (Eastman-Kodak, Rochester, NY) in 400 µl polyethylene centrifuge tubes. Cells and bound ligand were separated from free ligand by centrifuging the tubes for 1 min in a Beckmann microfuge at 8°C and cutting the bottom off the tube with a razor blade. Top and bottom segments of the tubes were placed in 12- x 75-mm glass tubes and counted separately on a Cobra gamma counter for 1 min per sample.

The second type of intact cell radioreceptor assay used the MultiScreen™ assay (Millipore, Bedford, MA) plate filtration system. Cells and radiolabeled IL-1 at several concentrations of each reactant were incubated for 2 h in MultiScreen Durapore® (type HV, 0.45 µm pore size) filtration plates at 60 µl/well in a refrigerator (8°C) on a rocker plate. Nonspecific binding was measured at the two highest concentrations of radioligand, using 10 µg/ml of unlabeled IL-1α to block receptors. At the end of the incubation, the plate was placed on a MultiScreen vacuum filtration manifold, and the supernatants harvested into a 96-well microtiter plate. Supernatants were transferred to 12- x 75-mm tubes for counting. Cells were washed 3 times with ice-cold PBS by filtration (200 µl/well/wash), the medium removed, dried and harvested using a MultiScreen multiple punch apparatus, which simultaneously delivers 8 samples into 8 separate 12- x 75-mm tubes for gamma-counting.

Binding of IL-1α and IL-1β to soluble murine IL-1 receptor was measured by an ELISA-style plate-binding assay, essentially as described by Dower et al. (9), with the exception that the Linbro plates were first coated with IL-1RM5 antibody (10 µg/ml in PBS), or the Fab fragment of IL-1RM5 (10 µg/ml in PBS) rather than polyclonal rat anti-receptor antiserum.

Binding of IL-1α to detergent-solublized full-length IL-1 receptor was measured by first binding the receptor to antibody-Affigel conjugates, and subsequently using the Affigel-antibody/receptor affinity matrix as a substrate for ligand binding. Five hundred µl aliquots of IL-1RM5-Affigel slurry (50% packed gel by volume) were added to 10-, 1- or 0.1-ml aliquots of receptor-containing Triton X-100 extract, at a receptor concentration of 3 x 10^{-9} M. The presence of the Triton was previously shown not to interfere with the binding assay (24). The mixtures were incubated overnight at 8°C, and subsequently the affinity matrix was washed 3 times with PBS and resuspended as a 50% slurry. For the binding assay, the antibody/receptor gel was used either undiluted or diluted 10, 100 or 1000 fold prior to addition. In each case 50 µl of gel suspension were added to 100 µl of radioligand solution; controls contained, in addition, 10 µg/ml unlabeled IL-1α. The matrix was incubated with ligand for 2 h at 8°C in MultiScreen

filtration plates, and unbound ligand subsequently removed by filtration as above. The matrix was washed three times with PBS and harvested as described for cells.

The data for all the assays were reduced from cpm to concentrations of bound and free ligand, analyzed by non-linear least squares fitting and graphed, using RS/1 (Bolt, Beranak and Newman), a data analysis package running on a VAX 11/780 under VMS.

RESULTS

Binding of IL-1 to Intact Cells

Figure 1 shows binding of radiolabeled IL-1α to EL-4 6.1 c10 cells measured by both the phthalate oil method (closed symbols) and the MultiScreen plate filtration method (open symbols). Each assay was done at four different cell concentrations (3.3 x, 1.7 x, 0.83 x and 0.42 x 10^7 cells/ml). At the highest cell concentration, the filtration assay failed because the filtration rate was so slow that the assay became impractical. Thus, with this cell line and membrane pore size, there is a limit of 1 million cells/well to the filtration assay system, presumably due to physical obstruction of the filter. Another reported IL-1 filtration assay utilized 1 x 10^6 cells/well and a 5 µm-pore-size Durapore membrane (14). Although the 5 µm pore-size membrane was not evaluated at 2 million cells/well, the membrane's inherently faster flow rate would perhaps yield a practical assay at this increased cell density. By contrast, the phthalate oil centrifugation assay failed at the lowest concentration of cells used. The optimal cell density utilized in the phthalate oil assay is particularly dependent on the cell line and its origin, that is, human vs. mouse (1,7,8,18). The oil assay relies on the use of an oil mixture which can be adjusted in composition and, hence, in density, so that it is more dense than aqueous medium, but less dense than cells. In principle, once this density range is achieved, the cell concentration should not affect recovery of cells at the bottom of the tube. In practice, however, the surface tension at the oil-water interface places a lower limit on the cell concentration that can be used in the assay, since single cells are insufficiently massive to penetrate this barrier. Separation is achieved by accumulation of the cells at the oil-water interface, forming a three-layer system that is unstable due to the cell layer density. The cell layer then breaks up and cells pass through the oil layer in clumps. Presumably it requires a minimum mass of cells per tube for this to occur. Comparison of the two assays over a range of cell concentrations suggests that in a situation where only a limited number of cells are available, the filtration method will be superior; conversely, where cells or tissue is not a limiting factor but where the signal is very weak, either because of low specific-activity ligand or low binding-site levels on the cells, the centrifugation method may be a better assay.

Quantitative analysis of the three data sets from each assay that yielded analyzable data was done by nonlinear least squares fitting of a one-site non-cooperative binding model, as described previously for the IL-1 receptor system (8). The resulting parameter values are given in Table 1. Inspection of the values shows that both assays perform similarly in terms of affinities and site numbers and the error values associated with those estimates (a measure of the scatter in the data used to derive the values). There is some tendency for the binding constants estimated from the Multiscreen data to be 1.5 to 2 fold higher than those estimated from the oil assay data. The reason for this is unclear, but it may be due to washing off of some weakly bound ligand in the plate assay. This would not occur with the oil centrifugation method, since the IL-1 is not soluble in the oil mixture. The oil assay was developed for weak binding ligand with fast dissociation rates, and clearly any method which employs aqueous washing will be of limited use for such systems. The IL-1 receptor system shows very slow dissociation kinetics (8,24), and hence does not suffer from this limitation. Finally, the backgrounds and signal-to-noise ratios in the two assays are essentially identical

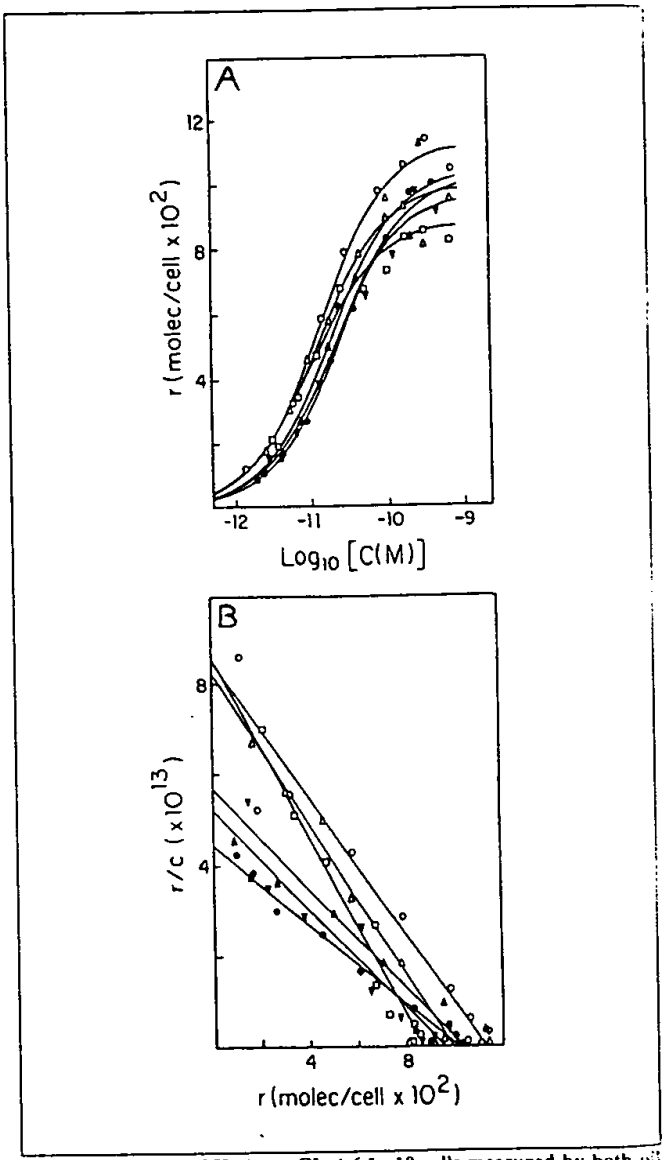

Figure 1. Binding of IL-1α to EL-4 6.1 c10 cells measured by both oil centrifugation and MultiScreen assays. Binding of ^{125}I-IL-1α to EL-4 6.1c10 cells was measured at various radioligand concentrations, using either the oil centrifugation assay (filled symbols) or the MultiScreen assay (open symbols). Data were collected at 3.3 x 10^7 (▲), 1.7 x 10^7 (●), 0.8 x 10^7 (▼) cells/ml for the oil centrifugation assay; and 1.7 x 10^7 (○), 0.8 x 10^7 (△), 0.4 x 10^7 (□) cells/ml for the MultiScreen assay. Nonspecific binding (NSB) was measured in the presence of 10 µg/ml of unlabeled IL-1α, and has been subtracted from the data prior to analysis and presentation. The values for NSB are given in Table 1. The continuous curves passing through the data are calculated using a simple one-site binding model and the parameters given in Table 1. Panel A shows the direct binding data, panel B shows the same data set transformed into the Scatchard coordinate system.

Table 1. Comparison Between the Oil Centrifugation and MultiScreen Radioreceptor Assays for IL-1 Receptors

Oil Centrifugation Assay

Cells/ml ($\times 10^{-7}$)	Sites/cell ($\times 10^{-3}$)	K (M^{-1}) ($\times 10^{-10}$)	NS (Molec/Cell/M) ($\times 10^{-10}$)	Signal/ Noise*
3.3	1.04 ± 0.05	5.5 ± 1.2	4.82	1140
1.7	1.02 ± 0.02	4.4 ± 0.5	4.92	912
0.8	0.96 ± 0.04	5.4 ± 1.0	4.36	1190
0.4	ND	ND	ND	ND

MultiScreen Assay

Cells/ml ($\times 10^{-7}$)	Sites/Cell ($\times 10^{-3}$)	K (M^{-1}) ($\times 10^{-10}$)	NS (Molec/Cell/M) ($\times 10^{-10}$)	Signal/ Noise*
3.3	ND	ND	10	ND
1.7	1.13 ± 0.02	7.5 ± 0.7	5.0	1700
0.8	0.99 ± 0.04	8.3 ± 1.6	7.6	1080
0.4	0.88 ± 0.03	9.8 ± 1.6	14	616

Parameter values were estimated from the data in Figure 1 by nonlinear least squares fitting of a simple non-cooperative single site binding model (8).

*The value for signal to noise is calculated as ([sites/cell] x K/NSB), which is the ratio of the slopes of the specific and nonspecific binding curves at the limit of zero receptor occupancy.

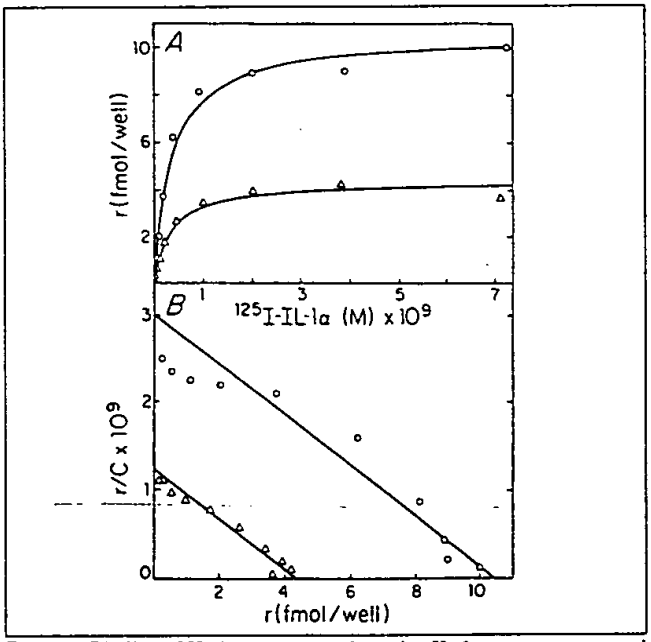

Figure 2. Binding of IL-1α to truncated murine IL-1 receptor measured with a plate binding assay. Binding of ^{125}I-IL-1α to soluble murine IL-1 receptor was measured at various radioligand concentrations, using plates coated with IL-1RM5 (10 µg/ml) (○) or its Fab fragment (10 µg/ml) (Δ) and subsequently with truncated soluble murine IL-1 receptor (residues 1-316 [9]) (100 ng/ml). Nonspecific binding measured in the presence of 10 µg/ml of unlabeled IL-1α was less than 5 x 10^6 fmol/well/M. The continuous curves passing through the data are calculated using a simple one-site binding model and the parameters given in the text. Panel A shows the direct binding data, panel B shows the same data set transformed into the Scatchard coordinate system.

(Table 1). Previous work (21) has demonstrated the extremely low nonspecific binding of radiolabeled hormones and proteins to the Durapore membranes. Horuk (14) has specifically shown that IL-1 has much lower non-specific binding to Durapore compared to both glass fiber and nitrocellulose filters.

Cell-Free IL-1 Binding Assays

Figure 2 shows the binding of ^{125}I-IL-1α to soluble murine IL-1 receptor (residues 1-316), using a plate binding assay, in which ELISA plates are coated with IL-1RM5 antibody, either the intact IgG or the Fab fragment, and subsequently with soluble receptor. The coated plates are then used to bind radiolabeled IL-1α in a standard 2 h 8°C incubation using an acid wash (pH 3.0) to recover bound ligand in the supernatant. The data show that whether plates are coated with the whole antibody or the Fab fragment, the antibody/receptor complexes bind IL-1α with the same affinity: 2.9 ± 0.3 x 10^9 M^{-1} for the IgG coated plates, and 2.8 ± 0.3 x 10^9 M^{-1} for the Fab-coated plates. The saturation binding levels, however, are somewhat different: 10.5 ± 0.4 fmol/well for the IgG as opposed to 4.4 ± 0.2 fmol/well for the Fab. The affinity is within the range reported for intact cells (23,24). It is clear from binding of IL-1RM5 to intact cells that the antibody can bind bivalently and that the affinity of the Fab fragment for the receptor is 6 x 10^8 M^{-1} (2). Under the conditions used in the experiment shown in Figure 2A, therefore, the receptor (2 x 10^{-9} M) should produce about 60% receptor occupancy with 36% doubly bound IgG molecules and 48% singly occupied. The finding suggests that dimerization of receptor has no impact on ligand binding. This is consistent with two other observations: that varying receptor density on the plates at constant antibody does not change the measured affinity, and that by gel filtration the soluble IL-1 receptor and IL-1 form a complex containing only one molecule of each reactant (9). The affinities measured in the experiment in Figure 2 are somewhat lower than those measured for the intact cells (Table 1). The values reported for the intact cells are unusual, being at the high end of the range for those we have observed for this system. The final assay format we employed in these studies uses an IL-1RM5 affinity matrix to capture IL-1 receptor, creating a receptor solid phase. The receptor-coated beads are subsequently used in an analogous fashion to the receptor-coated plates, placing the beads in MultiScreen plates and adding ligand to the wells. Parenthetically, it should be noted that attempts to couple the purified soluble receptor protein to Affigel have shown that 99% of the coupled receptor is incapable of binding IL-1: hence the need for the antibody as an intermediate link (S. Dower, unpublished observations). In order to explore a wide range of conditions for the assay, we coated the affinity matrix at three different levels of receptor using 30.0, 3.0 and 0.3 pmol receptor total for 500 µl of antibody matrix. The receptor matrices thus produced were assayed at 3, 30, 300 or 3000 fold final dilutions in the assay. The data generated from the lowest matrix concentration showed such low levels of bound ligand that no analysis could be performed. At the higher concentrations, however, two of the three loading levels produced matrices that showed detectable binding. At the lowest loading level (0.3 pmol/receptor), insufficient binding was detected to make analysis practical. The results of the analysis of the data collected at 30 pmol (1/3, 1/30, 1/300) and 3 pmol (1/3, 1/30, 1/300) are shown in

Table 2. Binding of IL-1 to Solubilized IL-1 Receptor using an Affinity Matrix Multiscreen Assay

pmol*	Dilu	fmol/well	K (M^{-1}) ($\times 10^{-9}$)	NS (fmol/well/M) ($\times 10^{-8}$)	Signal/ Noise*
30	3	193 ± 42	1.0	5.8	333
30	30	11.2 ± 3.6	1.4 ± 0.6	2.4	65
30	300	1.4 ± 0.7	1.3 ± 1.0	2.5	7.3
3	3	40 ± 29	1.6 ± 1.6	1.6	400
3	30	2.3 ± 0.4	8.0 ± 2.0	2.8	66
3	300	0.7 ± 0.3	4.5 ± 3.9	2.1	15

Parameter values were estimated from the data in Figure 1 by nonlinear least squares fitting of a simple non-cooperative single-site binding model (8).

*The value for signal to noise is calculated as ([sites/cell] x K/NSB), which is the ratio of the slopes of the specific and non-specific binding curves at the limit of zero receptor occupancy.

Figure 3. The system shows, at maximum loading, a higher level of specific binding than the plate binding assay, *ca.* 10-20 fold higher. However, the nonspecific binding is higher and signal-to-noise somewhat lower than in the plate assay (Figure 2 and Table 2). Since the filters in the MultiScreen plates do not show any significant level of nonspecific binding (14,21), the Affigel support must be the source of the background. The problem may be resolved by changing solid supports.

The binding constants for IL-1 to the receptor are comparable, and there is the expected dose-response curve for maximal amount bound as a function of receptor loading.

CONCLUSIONS

This report describes four quite different assay formats for analyzing the interactions between IL-1 and its receptor. Each

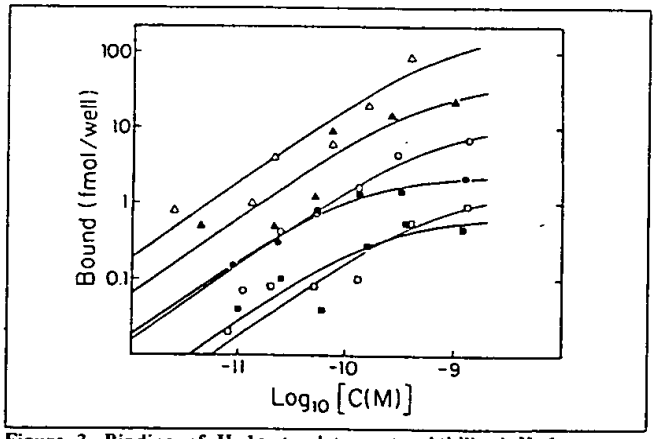

Figure 3. Binding of IL-1α to detergent solubilized IL-1 receptor measured using an affinity matrix solid-phase MultiScreen assay. Binding of ^{125}I-IL-1α to detergent solubilized full-length murine IL-1 receptor was measured at various radioligand concentrations using IL-1RM5 Affigel that had been precoated at 30 pmol receptor per 500 µl of gel (open symbols) or 3 pmol receptor per 500 µl of gel (closed symbols). Prior to assay the washed, receptor-coated gel was diluted 1/100 (□, ■), 1/10 (○, ●) or was used undiluted (△, ▲). Affigel with bound ligand was collected using the MultiScreen system. Nonspecific binding was measured in the presence of 10 µg/ml of unlabeled IL-1α. The values are given in Table 2. The continuous curves passing through the data are calculated using a simple one-site binding model and the parameters given in Table 2.

assay approach has different potential applications. The oil centrifugation and MultiScreen plate filtration assays for binding of IL-1 to intact cells yield comparable data and can both, presumably, be applied to a wide range of receptor systems. The major practical difference between them, as revealed by the data collected in the current set of experiments, is the cell concentration range over which each method can be applied. The oil assay requires relatively large numbers of cells, whereas the filter plate cannot be used at high cell concentrations, and therefore may be difficult to use in systems where receptor numbers are limited. It should be pointed out, however, that the cells assayed in these experiments had relatively low receptor numbers (*ca.* 10,000/cell), so that in real terms there may be few systems for which this is a significant problem. In terms of applied research, the Multiscreen system offers the possibiity of designing a relatively rapid throughput screen, an important consideration where compound screening by radioreceptor assay is contemplated.

The other two assay formats exploit the robustness of the IL-1/receptor interaction. It has been previously shown that IL-1 receptor can be extracted from cells in detergent, adsorbed to nitrocellulose, and retain its ligand binding properties. Furthermore, a polypeptide composed of only the extracellular domain of the receptor has been shown to possess ligand binding properties indistinguishable from the full-length integral membrane protein. This receptor fragment, when expressed in recombinant form, is secreted into the medium as a soluble protein which retains the IL-1 binding properties of the intact receptor. The radioligand solid-phase immune complex binding assay (RIBA) exploits a nonblocking monoclonal antibody to create a receptor-coated plate which binds ligand. This assay is an excellent analytical tool but has a low saturation level (10 fmol/well). The affinity matrix-based MultiScreen assay offers an alternative with some potential additional applications. Foremost, it has a higher saturation level (100-200 fmol/well) and with further optimization might be capable of being pushed higher. This method, which creates an affinity matrix containing large amounts of full-length receptor, could also potentially be used to study the functioning of the whole molecule, the cytoplasmic region, for example. Both methods can be used for a convenient screening assay, although again the affinity matrix/MultiScreen method has an advantage that it uses crude extracts of cells rather than purified recombinant receptor fragment. In summary, this report illustrates, within the context of a single receptor system, that a variety of assay formats can be designed for studying a ligand-receptor interaction. The choice of assay method for any given system and application will be dependent on the nature of the application. We have described the kind of criteria that should be used to determine which method might best be applied to any given problem.

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